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## Mutational analysis and chemical modification of Cys24 of lactococcin B, a bacteriocin produced by *Lactococcus lactis*

Koen Venema,<sup>†</sup> Michiel H. R. Dost, Gerard Venema and Jan Kok

Author for correspondence: Gerard Venema. Tel: +31 50 3632092. Fax: +31 50 3632348.

Department of Genetics,  
Groningen Biomolecular  
Sciences and Biotechnology  
Institute, University of  
Groningen, Kerklaan 30,  
9751 NN Haren, The  
Netherlands

**Using site-directed mutagenesis the single cysteine residue at position 24 of lactococcin B was replaced by all other possible amino acids. Most of these mutant molecules retained bacteriocin activity, with the exception of those in which cysteine was replaced by a positively charged amino acid. This would seem to be in agreement with the authors' earlier observation that treatment of the wild-type molecule with HgCl<sub>2</sub> resulted in its inactivation. The factor that causes inactivation of lactococcin B seems to be the introduction of a positive charge at position 24 by HgCl<sub>2</sub> rather than oxidation of this residue, as treatment of the bacteriocin with other oxidative chemicals did not interfere with the ability of lactococcin B to dissipate the membrane potential of sensitive cells. Results are also reported which imply that inactive lactococcin B can still bind to its receptor. It can be replaced by an active bacteriocin molecule, resulting in dissipation of the membrane potential.**

**Keywords:** lactococcin, secretion, bacteriocin, *Lactococcus lactis*

### INTRODUCTION

In the past several years there has been an explosion of research activity on bacteriocins produced by lactic acid bacteria (Klaenhammer, 1988, 1993; Jung & Sahl, 1991; De Vuyst, 1993; Hoover & Steenson, 1993; Nettles & Barefoot, 1993). As this field continues to expand, our knowledge about lactic acid bacteria and their bacteriocins is increasing rapidly, owing to detailed studies of the peptides, their mechanism of action, and the immunity, processing and secretion systems involved. Lactococcins are bacteriocins produced by *Lactococcus lactis*. The genes specifying production of lactococcins A, B and M, and their immunity and secretion are located on plasmid p9B4-6 and have been sequenced (Van Belkum *et al.*, 1989, 1991a, 1992). Lactococcins A and B specifically inhibit the growth of lactococci, and their modes of action have been studied. Both are small cationic, hydrophobic peptides that structurally resemble several peptide antibiotics which permeabilize membranes (Kordel & Sahl, 1986; Kordel *et al.*, 1988; Schaller *et al.*, 1989; Galvez *et al.*, 1991; Gao *et al.*, 1991). At lactococcin concentrations that do not affect immune cells, both bacteriocins rapidly dissipate the membrane potential of glucose-energized

sensitive cells of *L. lactis* and cause efflux of pre-accumulated amino acids (Van Belkum *et al.*, 1991b; Venema *et al.*, 1993). Efflux is a direct consequence of permeability changes in the cytoplasmic membrane, and is not caused by the dissipation of the proton-motive force. Both lactococcins are able to permeabilize the cytoplasmic membrane in a voltage-independent manner, most likely by the formation of pores. It is believed that both lactococcins require a proteinaceous receptor for their insertion into the cytoplasmic membrane (Van Belkum *et al.*, 1991b; Venema *et al.*, 1993, 1994).

A prerequisite for lactococcin B activity is that its only cysteine residue (Cys24) is in the reduced state (Venema *et al.*, 1993). Partially purified (and thus oxidized) lactococcin B is almost inactive on whole cells. Only after addition of dithiothreitol (DTT; 5 mM) is lactococcin B capable of dissipating the membrane potential of sensitive whole cells. Cys24 in DTT-treated lactococcin B can be oxidized by HgCl<sub>2</sub>, resulting in the inactivation of the bacteriocin, while HgCl<sub>2</sub>-oxidized lactococcin B can be reactivated by DTT. It has been postulated that the reduced state of the Cys24 residue is required for receptor recognition or, alternatively, that the oxidized state of Cys24 changes the structure of the molecule in such a way that its insertion and/or pore-forming abilities are affected. In this study we have used site-directed mutagenesis together with chemical modification of Cys24 to investigate these possibilities.

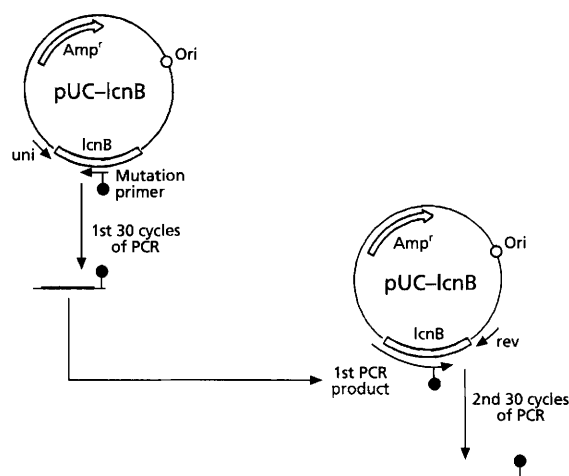
<sup>†</sup> **Present address:** Department of Food Science, North Carolina State University, Box 7624, Raleigh, NC 27695, USA.

## METHODS

**Bacterial strains, plasmids and growth conditions.** The bacterial strains and plasmids used are listed in Table 1. *L. lactis* was cultured in M17 broth (Terzaghi & Sandine, 1975) supplemented with 0.5% glucose (GM17) or in MRS broth (De Man *et al.*, 1960) containing 0.5% glucose. For solid plates, 1.5% (w/v) agar was used. *Escherichia coli* was cultured on TY medium and plates (Rottlander & Trautner, 1970). Erythromycin (Em) and chloramphenicol (Cm) were used at final concentrations of 5 µg ml<sup>-1</sup> for *L. lactis*, and 100 µg ml<sup>-1</sup> and 10 µg ml<sup>-1</sup>, respectively, for *E. coli*.

**Molecular cloning, DNA sequencing, and primer synthesis.** Transformation of *L. lactis* and *E. coli* was done as described earlier (Venema *et al.*, 1994). General DNA cloning and manipulation techniques were used essentially as described by Sambrook *et al.* (1989). DNA sequencing was done on double-stranded DNA by the dideoxy chain-termination method (Sanger *et al.*, 1977), using the T7 sequencing kit and protocol (Pharmacia). Synthetic oligodeoxynucleotides were synthesized with an Applied Biosystems 381A DNA synthesizer. The sequences of the primers were as follows (the position of degenerate nucleotides is indicated in parentheses; the position corresponding to that of the cysteine residue is shown in *italic*; the *ScaI* sites in the primers are underlined): KOV10: 5'-GGTATAAAGATACTAGTACTGGAAA-AACAATAT-(TCAT)TAAACAGACAATTG-3'; KOV11: 5'-GGTATAAAGATACTAGTACTGGAAA-AACAATAT-(TCAG)(TAAACAGACAATTG-3'; KOV12: 5'-GGTATAAAGATACTAGTACTGGAAA-AACAATAA(TCAG)(TC)AAACAGACAATTG-3'; KOV13: 5'-GGTATAAAGATACTAGTACTGGAAA-AACAATAG(TCAG)(TG)AAACAGACAATTG-3'; KOV14: 5'-GGTATAAAGATACTAGTACTGGAAA-AACAATATGGAAACAGACAATTG-3'.

**Bacteriocin assay.** Overlays on colonies were done as follows: colonies were treated with chloroform vapour for 15 min. After subsequent exposure of the plates to air for 30 min, M17 soft agar (0.75%) seeded with 0.1% of an overnight culture of the indicator strain *L. lactis* IL1403 was poured onto the plates. After 12–14 h incubation at 30 °C, the plates were examined for



**Fig. 1.** Two-step PCR strategy to change the *lcnB* codon at position 24. For details of the procedure see Methods. Amp<sup>r</sup>, ampicillin resistance marker; Ori, origin of replication; uni, universal primer; rev, reverse primer; †, position of the mutation.

zones of growth inhibition (haloes). The sizes of the haloes were measured from the edge of a bacteriocin-producing colony to the edge of the inhibition zone.

**Construction of mutants of lactococcin B.** The codon for Cys24 in the lactococcin B gene was changed, using a two-step PCR strategy, into codons for all other possible amino acids. For this, four degenerate primers were used. One primer (KOV14) was used to replace Cys24 by tryptophan. The lactococcin B operon from pMB580 was cloned in pUC19 as an *EcoRI*–*XbaI* fragment to give pUC-lcnB. This plasmid was used as a template in the first PCR step using the mutation primers and the universal primer (Boehringer Mannheim) (Fig. 1). The products obtained in the five different reactions were purified by Qiaprep PCR columns (Qiagen) and used as primers together with the reverse primer (Boehringer) on pUC-lcnB in a second PCR step (Fig. 1). The purified PCR products were restricted with the enzymes *EcoRI* and *XbaI* and cloned in pUC19. The ligation mixture was used to transform *E. coli* and the appropriate constructs were selected by screening for the presence of a *ScaI* site, introduced by the mutation primers. The *ScaI* site does not introduce amino acid change in lactococcin B. Inserts containing a *ScaI* site were sequenced to determine which mutation they contained and transferred to pMG36CT using the *EcoRI* and *XbaI* sites. The set of 19 mutants and the wild-type gene, all in pMG36CT, were introduced in *L. lactis*, which was subsequently screened for bacteriocin activity in a colony-overlay assay.

**Measurement of cytoplasmic membrane potential.** The change in cytoplasmic membrane potential of cells of *L. lactis* was monitored by following the distribution of the lipophilic cation tetraphenylphosphonium (TPP<sup>+</sup>) as described previously (Venema *et al.*, 1993).

**Chemical modification of lactococcin B.** Modification of Cys24 was performed with the following chemicals: cysteine, *N*-ethylmaleimide, *p*-mercuribenzoate, performic acid, CuSO<sub>4</sub> and HgCl<sub>2</sub>. Conditions were optimal for reaction of only the thiol group with the oxidative chemicals and are described in Means & Feeney (1971). The exception was performic acid, which, under these conditions, also reacts with methionine. To examine

**Table 1.** Bacterial strains and plasmids

Strain or plasmid	Relevant properties	Source or reference
<b>Bacteria</b>		
<i>L. lactis</i> IL1403	Plasmid-free, indicator strain for lactococcin B	Chopin <i>et al.</i> (1984)
<i>E. coli</i> JM103		Yanisch-Perron <i>et al.</i> (1985)
<b>Plasmids</b>		
pMB580	Em <sup>r</sup> , contains the lactococcin B operon	Van Belkum <i>et al.</i> (1992)
pUC19	Amp <sup>r</sup> , cloning vector	Yanisch-Perron <i>et al.</i> (1985)
pMG36CT	Cm <sup>r</sup> , cloning vector	C. M. Franke*
pUC-lcnB	Amp <sup>r</sup> , contains the lactococcin B operon	This work

\* Genetics Dept, University of Groningen.

whether the chemicals had reacted with the cysteine, the molecule was subsequently treated with  $\text{HgCl}_2$ , which would inactivate the bacteriocin even when it had not reacted with the other oxidative chemicals. These data indicated that all the chemicals had reacted with Cys24 (data not shown).

## RESULTS

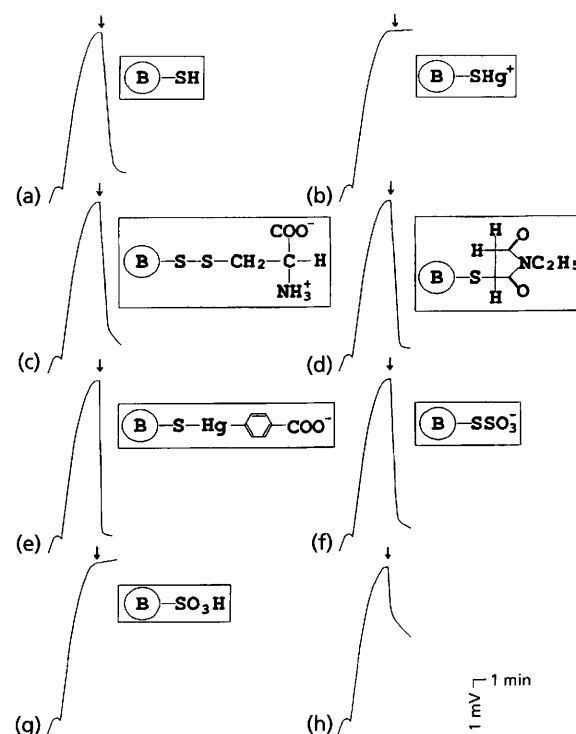
### Cys24 is not essential for lactococcin B activity

Using a two-step PCR procedure with degenerate primers, the gene encoding lactococcin B was mutated in such a way that Cys24 was replaced by one of the other 19 amino acids. The mutated variants of *lcnB* together with the lactococcin B immunity gene, *lciB*, were introduced into *L. lactis* IL1403 and the transformants were tested for lactococcin B activity in a colony-overlay assay. As shown in Table 2, all mutants produced active bacteriocin, at similar levels to the wild-type (data not shown), as judged by the presence of a halo around the colonies, with the exception of those mutants in which Cys24 had been replaced by a positively charged residue: Arg24, His24 or Lys24. Apparently, a cysteine residue at position 24 is not required for lactococcin B to be active. All active mutants displayed a higher bacteriocin activity than the wild-type, which can be explained by the fact that in the wild-type bacteriocin Cys24 is highly oxidized in the plate assay (Venema *et al.*, 1993). All strains were also immune to the mutant lactococcin B they produced except for the strain producing lactococcin B(Thr24), the colonies of which were initially very small. It appeared that the plasmid contained one extra mutation: a stop was introduced in the fourth codon of the immunity gene by one base substitution (data not shown). The number of transformants obtained with the plasmid carrying the mutated immunity gene was the same as that obtained with a plasmid carrying a functional immunity gene (data not shown).

**Table 2.** Activity of lactococcin B variants mutated at residue 24

Activity was measured (in triplicate) as a zone of inhibition (halo) around a colony producing bacteriocin for 18 h at 30 °C using IL1403 as the indicator strain. —, No halo; +, halo 1–6 mm; ++, halo 6–12 mm; +++, halo 12–18 mm.

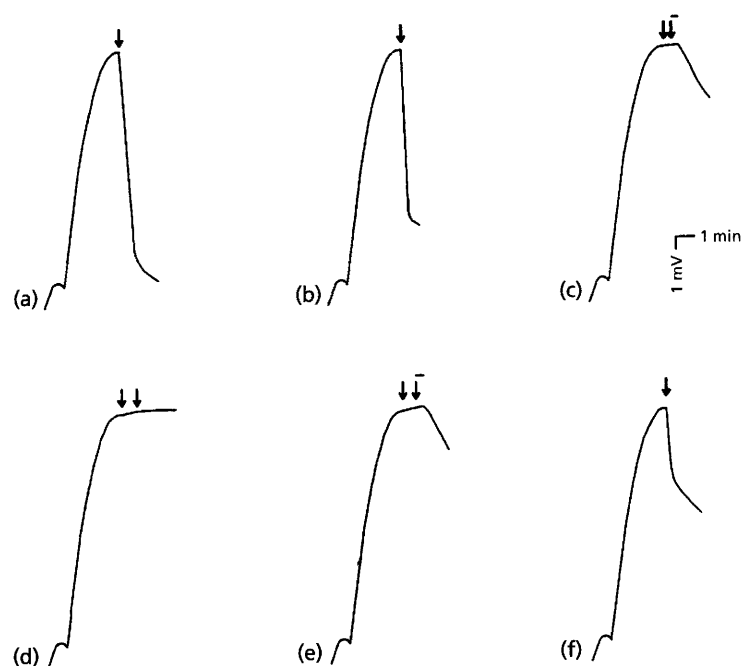
Mutant	Activity	Mutant	Activity
Cys (wild-type)	+		
Ala	+++	Lys	—
Arg	—	Met	++
Asn	++	Phe	++
Asp	++	Pro	++
Gln	++	Ser	++
Glu	++	Thr	++
Gly	++	Trp	++
His	—	Tyr	++
Ile	++	Val	++
Leu	++		



**Fig. 2.** Effect of lactococcin B treated with various chemicals on the membrane potential of energized cells of *L. lactis* IL1403. All curves have been normalized to that of the positive control, addition of valinomycin (h). The chemical bonds formed upon oxidation are shown in the boxes. Lactococcin B was treated with dithiothreitol (a),  $\text{HgCl}_2$  (b), cysteine (c), *N*-ethylmaleimide (d), *p*-mercuribenzoate (e),  $\text{CuSO}_3$  (f), and performic acid (g). Treated lactococcin B was added when the membrane potential had reached its optimum (at the time indicated by the arrow). Differences between the final membrane potential of two different curves after dissipation with lactococcin B were insignificant.

### A chemically introduced positive charge at position 24 abolishes lactococcin B activity

Lactococcin B can be inactivated by the oxidation of its only cysteine residue by  $\text{HgCl}_2$  (Venema *et al.*, 1993). Other oxidative chemicals specific for cysteine were tested to examine whether the lactococcin B molecule could be oxidized and still retain activity. The effect of the oxidized bacteriocin on the cytoplasmic membrane potential, as measured by the distribution over this membrane of the lipophilic cation tetraphenylphosphonium ( $\text{TPP}^+$ ), was tested. As documented previously, lactococcin B, when fully reduced by DTT, dissipates the membrane potential of sensitive cells of *L. lactis* (Fig. 2a). When lactococcin B is oxidized by  $\text{HgCl}_2$ , activity is lost completely (Fig. 2b). This can be explained by the acquisition of a positive charge at the position of Cys24 [see Fig. 2b, box; chemical structures derived from Means & Feeney (1971)] and is in agreement with the observation that lactococcin B mutants, in which Cys24 is replaced by a positively charged amino acid, are inactive (see above). To examine whether lactococcin B could be oxidized with other chemicals



**Fig. 3.** Effect of various lactococcin B derivatives on the membrane potential of energized cells of *L. lactis* IL1403. All curves have been normalized to that of the positive control, addition of valinomycin (f). Additions (at the time indicated by the arrow) were: lactococcin B(Ala24) (a); HgCl<sub>2</sub>-treated lactococcin B(Ala24) (b); HgCl<sub>2</sub>-inactivated lactococcin B (first arrow) followed by DTT-treated lactococcin B (second arrow) (c); HgCl<sub>2</sub>-inactivated lactococcin B (first arrow) followed by DTT (second arrow) (d); HgCl<sub>2</sub>-inactivated lactococcin B (first arrow) followed by lactococcin B(Ala24) (second arrow) (e); valinomycin as the positive control (f). The bars in (c) and (e) indicate the lag time between addition of lactococcin B and the start of the membrane potential dissipation. Differences between the final membrane potential of two different curves after dissipation with lactococcin B were insignificant.

without introducing a positive charge, the bacteriocin was reacted with cysteine, *N*-ethylmaleimide, *p*-mercuribenzoate, or CuSO<sub>3</sub>. As can be seen in Fig. 2(c-f), none of these oxidative compounds affected lactococcin B activity. The chemical bond that is formed upon oxidation is shown in the boxes of these figures. These results indicate that it is the presence of a positive charge at this position that abolishes bacteriocin activity. Treatment of lactococcin B with performic acid resulted in loss of activity (Fig. 2g), although in this case no positive charge was introduced at position 24 (see box in Fig. 2g). Also, the lactococcin B molecule containing the Ala24 substitution was inactivated by performic acid (data not shown), indicating that the inactivation was not caused by oxidation of the cysteine residue.

#### Lactococcin B(Ala24) dissipates the membrane potential of sensitive cells

The effect of one of the lactococcin B mutants on the membrane potential was investigated. The Ala24 mutant was chosen, since it produced the largest halo in the overlay assay (see Table 2). Fig. 3(a) shows that lactococcin B(Ala24) dissipated the cytoplasmic membrane potential of sensitive cells. As expected, addition of HgCl<sub>2</sub> to lactococcin B(Ala24) had no effect on its activity (Fig. 3b) since this molecule no longer contains the oxidizable cysteine residue.

#### HgCl<sub>2</sub>-inactivated lactococcin B blocks the immediate action of subsequently added active lactococcin B

Approximately 30 s after HgCl<sub>2</sub>-inactivated lactococcin B was added to the cells (Fig. 3c, first arrow), active (DTT-treated) lactococcin B was added (Fig. 3c, second arrow). Instead of an immediate dissipation of the membrane

potential, there was a lag of approximately 25 s before dissipation started. Moreover, dissipation was much slower than when only active (DTT-treated) lactococcin B had been added (compare Figs 2a and 3c). To exclude the possibility that DTT caused this effect it was added to cells pretreated with HgCl<sub>2</sub>-inactivated lactococcin B. As can be seen in Fig. 3(d), DTT had no effect on the membrane potential of these cells. Apparently, dissipation of the membrane potential occurred as the result of the addition of the active bacteriocin and not because of re-activation of the HgCl<sub>2</sub>-inactivated molecules. This conclusion is in agreement with the fact that the active Ala24 mutant is still able to dissipate the membrane potential after the lag time of approximately 25 s (Fig. 3e). The Arg24 mutant also blocks immediate action of subsequently added lactococcin B (data not shown), similar to the effect observed with HgCl<sub>2</sub>-inactivated lactococcin B.

#### DISCUSSION

The two-step PCR procedure used to construct the mutants of lactococcin B produced the correct mutants at a frequency of 80%. In the other 20% of cases, extra mutations, such as frame-shift mutations and/or nucleotide substitutions, had been introduced. One example is the plasmid encoding lactococcin B(Thr24). In this plasmid, a stop was introduced in the fourth codon of the immunity gene by one base substitution. The transformants carrying this plasmid initially grew poorly, but their normal growth potential was gradually restored. This resumption of growth (tolerance) is frequently observed when sensitive cells are exposed to lactococcin (Van Belkum *et al.*, 1991a, 1992) and may be due to a change in lipid composition of their membranes (Ming & Daeschel, 1995).

We reported previously that lactococcin B is only active

when its single cysteine residue is in the reduced state (Venema *et al.*, 1993). Here we show that this cysteine can be replaced by all other amino acids, except the positively charged ones, without losing bacteriocin activity. Also, the bacteriocin molecule is still active when oxidized with oxidative chemicals other than  $\text{HgCl}_2$  (except performic acid). Clearly, Cys24 is not essential for bacteriocin activity. In this regard lactococcin B resembles a group of thiol-activated toxins in which the reduced state of the cysteine residue appears to be essential for the generation of functional lesions in toxin-treated membranes, but in which the cysteine residue can be replaced with other amino acids without loss of activity (Boulnois *et al.*, 1991). The fact that Cys24 can even be replaced by proline or glycine, two residues able to induce large structural changes, probably implies that this part of the molecule is not directly involved in the activity mechanism of the bacteriocin.

Addition of active lactococcin B to cells incubated with  $\text{HgCl}_2$ -inactivated lactococcin B results in a time lag before the onset of dissipation of the membrane potential. A possible explanation could be that the inactive bacteriocin is still able to bind to the bacteriocin receptor, and that active lactococcin B has to compete with the inactive molecules for receptor binding in a time-consuming process. Another possibility is that  $\text{HgCl}_2$ -inactivated molecules insert into the membrane, but form closed pores. Addition of active lactococcin B might then result in the formation of mixed pores consisting of both inactive and active lactococcin B molecules, which would open again when a certain ratio of inactive and active molecules is reached. If this interpretation is correct, the lag time would represent the time needed to reach the critical ratio of active to inactive molecules and would agree with the observation that the membrane potential dissipated more slowly than when only active lactococcin B was added. This might be due to the lower efficiency of solute transport through the mixed pores. At present it is not possible to distinguish between these two possibilities. Resolving this question must await the characterization of the lactococcin B receptor.

Treatment of lactococcin B with performic acid resulted in complete loss of bacteriocin activity, most probably due to the oxidation of the two methionine residues in the lactococcin B molecule, which are oxidized under the conditions used (Means & Feeney, 1971). One of the methionine residues is present in an amphiphilic helix (Met38; Venema, 1995). Oxidation of this residue would destroy the amphipathic nature of the helix. Alternatively, oxidation of the methionine residue at position 6 of the molecule could interfere with activity, for instance by blocking receptor binding.

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